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Immunological Survey of Planktonic Embryos and Larvae of the Starfish *Asterina pectinifera*, Obtained from the Sea, Using a Monoclonal Antibody Directed against Egg Polypeptides

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Abstract. A monoclonal antibody, K1, specifically recognizes polypeptides with apparent molecular masses of 56 and 58 kDa, in the egg of the starfish *Asterina pectinifera*. The K1 antibody reacted with extracts prepared from ovaries, oocytes, morulae, blastulae, gastrulae, and bipinnariae. Brachiolariae, testes, pyloric ceca, body walls, and tubefeet did not contain K1-reactive antigenic molecules. Extracts of eggs of the starfish *Asterias amurensis* and several sea urchin species did not react with the K1 antibody. Among the members of the genus *Asterina*, extracts of brachiolariae of *A. batheri* and *A. minor* were not reactive, whereas blastulae and brachiolariae of *A. pseudoexigua pacifica* did contain an antigenic component. Unlike the antigenic peptides in *A. pectinifera* eggs, the apparent molecular mass of the antigen molecule in embryos and larvae of *A. pseudoexigua pacifica* was 41 kDa, which represents a remarkable phylogenetic variation of antigen molecules. Using the K1 antibody, we have developed an assay system that detects embryos and larvae of *A. pectinifera* in complex mixtures of biological specimens obtained from the sea.

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Abbreviations: ASW, artificial seawater; BSA, bovine serum albumin; CaFSW, calcium-free seawater; D-MEM, Dulbecco's modification of Eagle medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HAT, hypoxanthine-aminopterine-thymidine; IgG, immunoglobulin G; kDa, kilodaltons; NSW, normal seawater; PBS, phosphate buffered saline without divalent cations; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; T·TBS, 0.5 *M* NaCl, 10 m*M* Tris-HCl (pH 7.8) and 0.05% Tween 20.

Introduction

The development of a number of starfish species has been described (Oguro and Komatsu, 1988). Nevertheless, the embryos and larvae obtained from the sea have hitherto been unidentifiable, because the entire process of their development and metamorphosis is not sufficiently well known. Furthermore, the identification of individual species by examination of external morphological features alone tends to be equivocal. Therefore, specific methods for detecting chemical components present exclusively in embryos and larvae of one species are needed.

We report the discovery of specific antigenic polypeptides that are present in eggs, embryos, and larvae of *Asterina pectinifera*, but not in those of other species belonging to the same genus. A single embryo or larva in a complex plankton mixture can be detected by immunological analysis with an enzyme-linked immunosorbent assay (ELISA) or immunoblot procedure, using a monoclonal antibody that recognizes the unique antigen molecules present in embryos and larvae.

Materials and Methods

Animals

Adults of the following starfish and sea urchin species were collected, during their breeding seasons, along the coast of the Japan Islands. The starfish were collected from the following areas: *A. pectinifera*—off Asamushi in Aomori Prefecture, off Otsuchi in Iwate Prefecture, off Hashirimizu in Kanagawa Prefecture, off Sensui Island

in Hiroshima Prefecture, and off Gogo Island in Ehime Prefecture; *Asterina pseudoexigua pacifica*—from the undersurface of stones at the intertidal zone of Kushimoto in Wakayama Prefecture; and *Asterina minor* and *Asterina batheri*—along the coast of the Noto Peninsula in Ishikawa Prefecture. The sea urchins, *Scaphechinus mirabilis, Hemicentrotus pulcherrimus,* and *Pseudocentrotus depressus,* were collected off Sensui Island in Hiroshima Prefecture, off Kiwado in Yamaguchi Prefecture, and off Sugashima Island in Mie Prefecture, respectively.

The plankton samples were collected by a diver equipped with SCUBA, who towed plankton nets (100 μm-mesh; open mouth, 40 cm in diameter) horizontally 50 m at discrete depths off Sensui Island in Hiroshima Prefecture.

Gametes, embryos, and larvae

Fertilizable eggs were induced to spawn from isolated ovaries of female *A. pectinifera* and *A. amurensis* by application of 1-methyladenine at a final concentration of 150 ng/ml (Kanatani, 1973). Spawned *A. pectinifera* eggs were washed several times with artificial seawater (ASW), Jamarin U (Jamarin Laboratory, Osaka), and inseminated at 40–60 min after the start of 1-methyladenine treatment. Formed embryos were cultured in ASW at 23° ± 2°C, and the early brachiolariae were reared in natural seawater (NSW), which was kept in the sun and changed every two or three days. Embryos of *A. batheri* were obtained similarly. Embryos and larvae of hermaphroditic *A. minor* were obtained from a container of adult colonies collected during the breeding season, during June at the Noto Peninsula in Ishikawa Prefecture. Embryos and larvae of *A. pseudoexigua pacifica* were collected from the body cavity of the adult during the breeding season, in July at Kushimoto in Wakayama Prefecture. Eggs of sea urchins were obtained by introducing 0.5 *M* KCl into the body cavity.

Eggs, embryos, and larvae were washed several times with ASW or NSW before being stored at −80°C until required.

Protein determination

Protein was determined by the protein-dye binding assay, with bovine serum albumin (BSA) as the standard (Bradford, 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with 5% stacking gel, as described by Laemmli (1970). The gels were then stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose sheets (Bio-Rad), as described below.

Preparation of antigens

Mature eggs (3 ml packed volume) from *A. pectinifera,* shed from the isolated ovaries of adults by application of 1-methyladenine at a final concentration of 150 ng/ml, were collected 1 h after the hormone treatment had started. They were washed, by settling, in 5 vol of ASW and homogenized, in a Potter-Elvehjem glass homogenizer, at 0°C in 12 ml 20 m*M* Tris-HCl (pH 7.5) buffer with 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF). The subsequent procedures were carried out at 4°C, unless otherwise stated. The homogenate was centrifuged at 18,000 × *g* for 20 min. The pellet was resuspended in 15 ml 20 m*M* Tris-HCl (pH 7.5) buffer with 0.1 m*M* PMSF, and the suspension was centrifuged again. The supernatants were combined, and solid ammonium sulfate was added to a final ammonium sulfate concentration of 20% (w/v). The mixture was stirred for 1 h, then centrifuged at 18,000 × *g* for 20 min. The supernatant was removed, the ammonium sulfate concentration was adjusted to 80% (w/v) by adding solid ammonium sulfate, and 1 h later, the mixture was centrifuged as before. The precipitate was removed and dissolved in 2 l loading buffer, composed of 50 m*M* Tris-HCl (pH 7.4) with 0.1 m*M* PMSF. The solution was then loaded onto a Whatman DE-52 ion-exchange resin column (2.5 × 3.3 cm), which had been equilibrated with loading buffer. Step-wise elution was performed with solutions of NaCl in loading buffer using 50 ml each of 20, 150, 200, and 500 m*M* concentrations. The fractions eluted with 150 and 200 m*M* NaCl were pooled and dialyzed against phosphate-buffered saline without divalent cations (PBS). The non-dialyzable fraction was frozen at −80°C for future use.

Preparation of nuclei

Two thousand, five hundred (2500) *A. pectinifera* embryos were washed twice with calcium-free seawater (CaFSW, Jamarin Laboratory, Osaka; 4°C) and once with buffer A (4°C), which consisted of 250 m*M* sucrose, 5 m*M* $MgCl_2$, 40 m*M* NaCl, and 50 m*M* Tris-HCl (pH 7.5). They were homogenized with a Dounce homogenizer at 4°C in 5 ml buffer A containing 1% (w/v) Triton X-100. The homogenate was passed through a 40-μm stainless steel filter by gravity. The filtrate was centrifuged at 1000 × *g* at 4°C for 5 min. The nuclear pellet was resuspended in 2.5 ml buffer A, followed by centrifugation at 1000 × *g* for 5 min. This process was repeated once. The washed nuclear preparations were frozen at −80°C for future use.

Monoclonal antibody production

A 7-week-old BALB/c female mouse was injected intraperitoneally with 100 μg protein in complete Freund's adjuvant. Four and then five weeks later, booster injections of 50 μg protein were administered. And three days after the second booster, the mouse was sacrificed, its spleen was removed, and the cells allowed to fuse, for 8 min, with myeloma cells (SP-2) (at a ratio of $1 \times 10^8/1 \times 10^7$ spleen cells/myeloma cells). The fusion was carried out in 1 ml Dulbecco's modification of Eagle medium (D-MEM), without serum, but containing 50% polyethylene glycol-6,000. To stop the fusion, 20 ml D-MEM was added drop-wise, and cell pellets were precipitated by centrifugation at $250 \times g$ for 5 min. The cell pellets were resuspended in 5 ml D-MEM containing 5% fetal calf serum (FCS, Microbiological Associates), incubated for 12–18 h, then plated in 98-well microtiter dishes at 200 μl/well in HAT medium (complete D-MEM containing 1×10^{-4} *M* hypoxanthine, 4×10^{-7} *M* aminopterin, 1.6×10^{-5} *M* thymidine, and 10% FCS). Half of the medium was removed from each well and replaced with fresh HAT medium after 2, 4, 7, and 10 days. The supernatants were harvested when the clones began to exhaust their medium and were screened by protein blotting, as described below. Hybridomas that showed reactivity were expanded and subcloned by limited dilution.

Hybridomas (1×10^7 cells) were injected intraperitoneally into a BALB/c mouse which, 1–2 weeks previously, at 8 weeks old, had received an injection of 0.5 ml 2,6,10,14-tetramethylpentadecane. One to two weeks after the injection of hybridomas, the ascites were collected and centrifuged at $1500 \times g$ for 30 min. The supernatant (1 ml) was dialyzed, at 4°C, against 50 m*M* Tris-HCl (pH 8.6) with 0.15 *M* NaCl. The non-dialyzable fraction was loaded onto a column (0.6 × 10 cm) of Protein-A cellulofine (Seikagaku Kogyo, Tokyo), which had been equilibrated with 50 m*M* Tris-HCl (pH 8.6) with 0.15 *M* NaCl. After washing with 30 ml 50 m*M* Tris-HCl (pH 8.6) with 0.15 *M* NaCl, the immunoglobulin G (IgG) fraction was eluted out by 0.05 *M* sodium acetate (pH 4.0) and 0.15 *M* NaCl. The pH of the IgG solution was adjusted to 7.2 by adding 1 *M* Tris-HCl (pH 9.0). The protein concentration was adjusted to 500 μg/ml, and then the IgG solution was frozen at −80°C for future use.

Immunoblotting

For screening hybridomas, nitrocellulose blots were prepared by electrophoretic transfer from preparative SDS-polyacrylamide gels of the 150 and 200 m*M* NaCl fractions obtained by Whatman DE-52 ion-exchange chromatography, performed as described above, followed by blocking in a solution of 10 m*M* Tris-HCl (pH 7.4), 0.5 *M* NaCl and 3% gelatin. Nitrocellulose strips (Bio-Rad) were incubated with undiluted culture supernatant at 37°C for 2 h with gentle stirring. After a brief wash with distilled water, they were then washed, for 10 min each, in two changes of excess 10 m*M* Tris-HCl (pH 7.4), 0.5 *M* NaCl and 0.05% Tween 20, and incubated for 2 h at 37°C with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) diluted 1:3000 in the same buffer that was used to block the nitrocellulose strips. After washing again, strips were assayed for peroxidase activity using H_2O_2 and 4-chloro-1-naphthol. The reaction was stopped by rinsing with water and strips were dried in air.

Protein blots of oocytes, eggs, embryos, and larvae were obtained by electrophoretic transfer from SDS-polyacrylamide gel to nitrocellulose sheets and then treated as specified above, except that the IgG solutions, diluted 1:100 to 1:1000 in PBS, were used for antibody incubations instead of hybridoma culture supernatant.

Immunofluorescence microscopy

Embryos were washed twice with ASW, fixed with 100% methanol for 10 min, immersed in 100% ethanol for 10 min and then embedded in polyester wax (BDH). Sections 7 μm thick were placed onto 0.1% (w/v) amylopectin-coated coverslips and incubated with IgG solution diluted 1:100 in PBS for 1 h, at room temperature, after which they were washed in PBS for 30 min. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago), diluted 1:200 in PBS containing 2% BSA was then added and the sections were incubated, in the dark, at room temperature, for 1 h, after which they were washed with PBS for 30 min. Coverslips were mounted in a solution of 20% glycerol in PBS, sealed, and viewed under a Nikon TMD-EP2 photomicroscope. Photographs were then taken using Kodak Tri-X pan ASA 400 film.

ELISA assay

Five thousand (5000) oocytes, eggs, or embryos were boiled for 2 min in 1 ml SDS sample buffer containing 62.5 m*M* Tris-HCl (pH 6.8), 2% SDS and 5% 2-mercaptoethanol (Laemmli, 1970). One hundred microliters of each of the extracts, at a dilution of 1:2000 in PBS, were added to a well of a microtiter plate for ELISA assay (Probind, Falcon), the content of the lysate in a well being equivalent to 0.25 oocytes, eggs, or embryos, and absorbed for 2 h at 37°C. The solution was removed, and the wells were treated at room temperature with 200 μl PBS with 1% BSA for 30 min. The IgG solution was diluted 1:1000 in PBS, a 100-μl aliquot was added to each well and incubated for 2 h at 37°C. The wells were washed twice with T · TBS buffer, composed of 0.5 *M* NaCl, 10 m*M* Tris-HCl (pH 7.4), and 0.05% (v/v) Tween 20, and then three times with distilled water. One hundred microliters of a solution of horseradish peroxidase linked to goat anti-

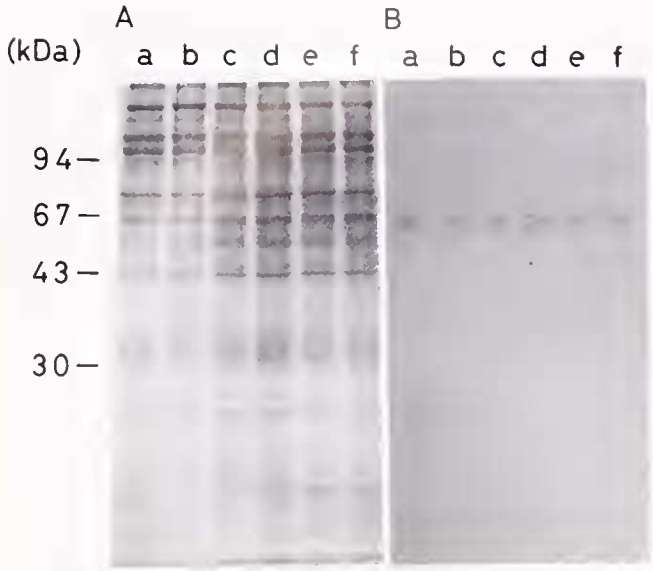

Figure 1. Immunoblots of *Asterina pectinifera* extracts. The extracts (5 μg protein each) were electrophoresed on SDS-12.5% polyacrylamide gels. Gels were either stained with Coomassie brilliant blue (A), or transblotted onto a nitrocellulose filter, which was probed with culture medium conditioned by a K1 antibody-producing hybridoma cell line (B). Lanes a, b, c, d, e, and f correspond to the extract of oocytes, eggs, 32-cell-stage morulae, 256-cell-stage blastulae, mid-blastulae (10 h after fertilization) and early gastrulae (24 h after fertilization), respectively.

mouse IgG, diluted 1:3000 in PBS with 0.05% Tween 20 and 1% gelatin, was added then incubated for 2 h at 37°C. The wells then were washed twice with T · TBS followed by three times with distilled water.

Two hundred microliters of substrate solutions containing H_2O_2 and *o*-phenylenediamine were added, left for 10–30 min, and then the reaction was stopped by the addition of 100 μl 4 N H_2SO_4. The absorbance at 492 nm was measured within 30 min of the sulfuric acid addition.

When the primary antibody was replaced with preimmune serum, or was omitted, the result was a completely negative one.

Results

Mice were immunized with the pooled 150 and 200 m*M* NaCl eluates obtained by Whatman DE-52 ion-exchange resin chromatography to produce monoclonal antibodies that would react with components present in *A. pectinifera* eggs, but not with any components of *A. amurensis* eggs. The spleen was dissociated from the immunized mice. The cells were fused to myeloma cells plated at a clonal dilution. From this fusion, 30 hybridoma clones grew. Hybridoma clones were selected by screening antibodies secreted in the media with the ELISA and immunoblot assays. Thirteen clones produced antibodies against *A. pectinifera* egg components with molecular masses of 188 or 133 kDa. All of these antibodies were found to bind to some components of *A. amurensis* eggs. One clone produced an antibody that recognized components of *A. pectinifera* eggs, but not those of *A. amurensis* eggs, in an ELISA assay. This specific antibody, K1, bound to *A. pectinifera* egg components with molecular masses of 58 and 56 kDa in an immunoblot assay (Fig. 1). The immunoreactive components were also present in extracts of *A. pectinifera* oocytes and embryos at the 32-cell morula, the 256-cell early blastula, mid-blastula (10 h after fertilization), and early gastrula (24 h after fertilization) stages. Protein A binding experiments indicated that the K1 antibody was of the IgG class.

The minimum number of eggs detectable by immunoblot analysis was then determined as follows. Graded amounts of egg lysate were loaded into slots of an SDS-polyacrylamide gel. After electrophoresis, the gel was blotted onto a nitrocellulose filter that was then probed with the K1 antibody. The lysate obtained from one sixteenth of an egg and loaded into a slot gave two bands at molecular masses of 58 and 56 kDa. The intensity of the bands was close to the limit of detection (data not shown).

To determine whether the antigenic molecules were polypeptides, we incubated 5 μg egg extract protein with 0.001 to 0.01 units of papain at 37°C for 30 min and subjected the reaction mixture to SDS-polyacrylamide gel electrophoresis. The gel was blotted onto a nitrocellulose filter, which was probed with the K1 antibody. The 58-

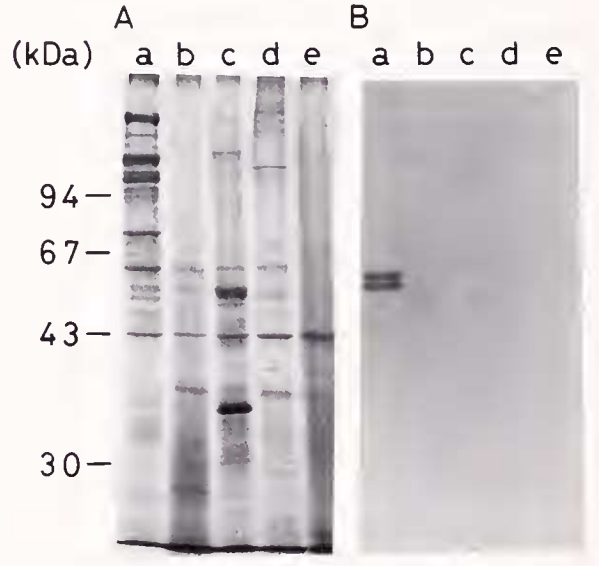

Figure 2. Immunoblots of tissues of adult *Asterina pectinifera*. The extracts (5 μg proteins each) were electrophoresed on SDS-10% acrylamide gels. Gels were either stained with Coomassie brilliant blue (A) or blotted onto nitrocellulose and probed with the K1 antibody (B), as described in Materials and Methods. Lanes a, b, c, d, and e correspond to extracts of ovary, pyloric cecum, testis, body-wall and tube-foot, respectively.

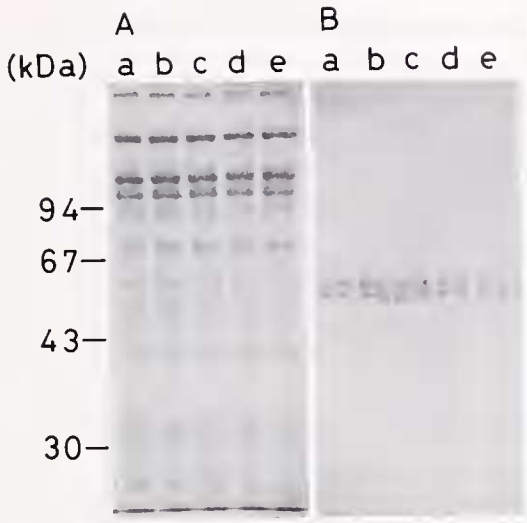

Figure 3. Immunoblots of extracts of *Asterina pectinifera* eggs and embryos. The extracts (5 μg protein each) were electrophoresed on SDS-10% polyacrylamide gels. Gels were either stained with Coomassie brilliant blue (A) or blotted onto nitrocellulose and probed with the K1 antibody (B) as described in Materials and Methods. Lanes a, b, c, d, and e correspond to the extract of eggs, 1-day-old early gastrulae, 2-day-old mid-gastrulae, 3-day-old late gastrulae and 4-day-old early bipinnariae, respectively.

and 56-kDa forms were no longer detectable on the immunoblot, and prominent degradation products with molecular masses of 49, 46, 43, and 40 kDa were observed (data not shown). Treatment of an identical amount of extract with a higher amount (0.1 unit) of papain resulted in total loss of immunoreactivity. These results demonstrate that the components recognized by the antibody were proteinaceous. The immunoreactive 58- and 56-kDa proteins were designated p58/56.

Distribution of the antigens in tissues, oocytes and embryos of A. pectinifera

Several tissues were excised from adult *A. pectinifera*, and their extracts were examined, by immunoblot analysis, to determine whether K1-reactive antigens were present. Antigenic p58/56 were found to be present in ovarian extracts of ovaries, whereas testes, pyloric ceca, and tube-feet contained no antigenic components (Fig. 2). Next, we surveyed extracts of hatched embryos and larvae by immunoblot analysis and found that p58/56 were present in all the samples examined; the amount of p58/56, on a protein weight basis, was almost constant throughout early embryonic development up to the early bipinnaria stage (Fig. 3).

Subcellular localization of the antigens in A. pectinifera *eggs and embryos*

Embryonic extracts were separated, by centrifugation, into nuclear and cytoplasmic fractions. Immunoblot analysis of these fractions showed that p58/56 were present in the cytoplasmic, but not in the nuclear fraction, as shown in Figure 4.

Embryos were fixed with methanol, embedded in polyester wax, sectioned, and the sections subjected to immunofluorescence staining. The K1 antibody stained the cytoplasm, but not the nucleus of an 8-cell-stage embryo (Fig. 5a). Figure 5b shows a section of a bipinnaria (4 days after fertilization) that was stained with the K1 antibody. Almost all of the embryonic cells contained some antigen molecules.

Occurrence of antigen molecules in other Asterina *species*

We attempted to survey the distribution of antigens in the eggs and embryos of some sea urchin and starfish species. Lysates were prepared from eggs of *S. mirabilis*, *P. depressus*, *H. pulcherrimus*, and *A. amurensis* and were subjected to immunoblot analysis; 10 μg of lysate were loaded into each slot. The analysis showed that no molecules reactive with the K1 antibody were present in these sea urchin and starfish eggs (Fig. 6).

Next, we examined embryos of species belonging to the genus *Asterina*: *A. minor*, *A. pseudoexigua pacifica*, and *A. batheri*. Development of these species is direct, with

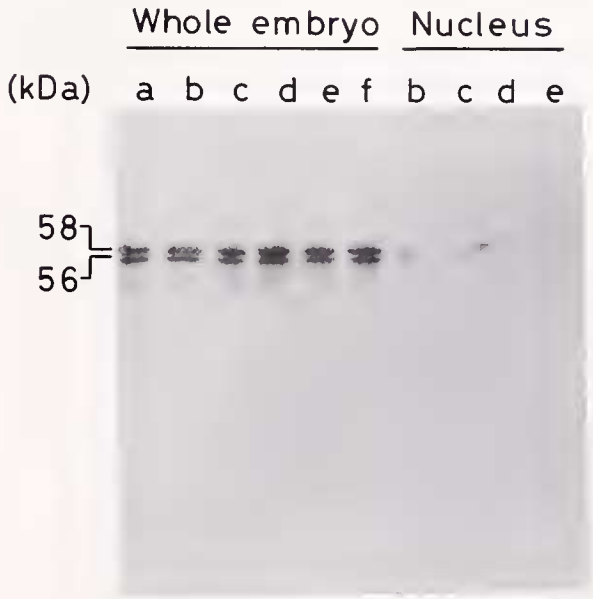

Figure 4. Immunoblots of whole and nuclear extracts of *Asterina pectinifera* embryos. The extracts (5 μg proteins each) were electrophoresed on an SDS-12.5% polyacrylamide gel. The gel was blotted onto nitrocellulose and probed with the K1 antibody, as described in Materials and Methods. Lanes a, b, c, d, e, and f correspond to the extracts at the 32-cell morulae, 256-cell early blastulae, 512-cell early blastulae, 1024-cell early blastulae, mid-blastulae (10 h after fertilization) and early gastrulae (24 h after fertilization), respectively.

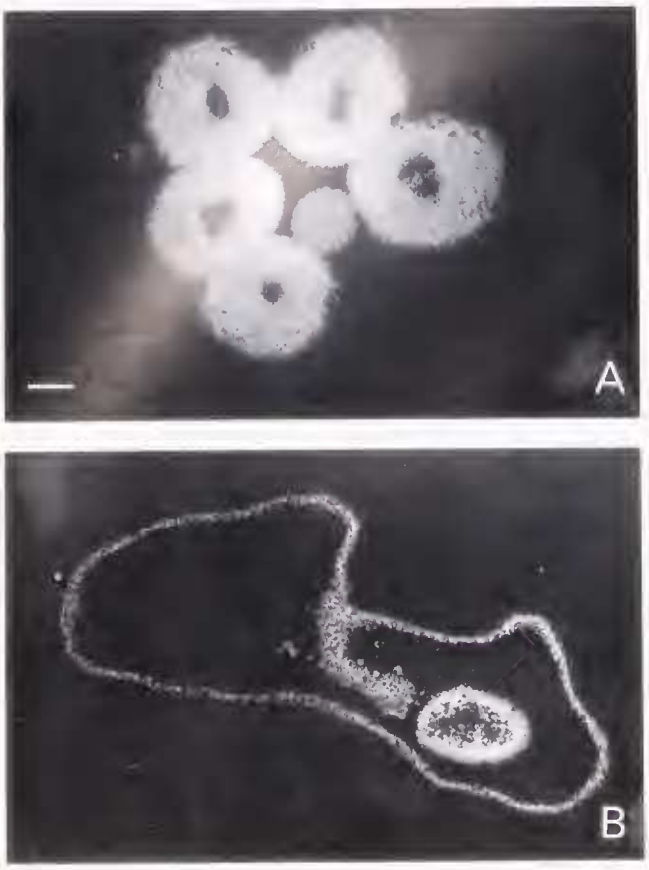

Figure 5. Immunofluorescence microscopy of sections of *Asterina pectinifera* embryos using the K1 antibody. A and B correspond to a 32-cell morula and an early bipinnaria, respectively. Bar indicates 100 μm. The magnification of B is the same as that of A.

no bipinnaria stage, unlike that of *A. pectinifera*. *A. minor* is hermaphroditic (Komatsu, 1976), and when spawning occurs, the animals cling to one another along the margins of their bodies, or they are imbricated with others (Komatsu *et al.*, 1979). Eggs that were spawned spontaneously and fertilized were cultured in the laboratory and allowed to develop into brachiolariae, which were then used for immunoblot analysis. *A. pseudoexigua pacifica* is ovoviviparous (Komatsu *et al.*, 1990), and we were able to collect early blastulae, wrinkled blastulae, and brachiolariae from the adult ovary. Brachiolariae of *A. batheri* (Kano and Komatsu, 1978) were obtained by rearing artificially inseminated, spontaneously spawned eggs. These embryos were boiled in SDS sample buffer and loaded onto a gel, which was electrophoresed and immunoblotted as described above. None of the peptides from the brachiolariae of *A. minor* or *A. batheri* reacted with the K1 antibody (Fig. 7). But when lysates of early blastulae, wrinkled blastulae, or brachiolariae of *A. pseudoexigua pacifica* were separated on a gel, a band at 41 kDa was detected on the blot (Fig. 8).

Detection of A. pectinifera *embryos in the ocean*

As *A. pseudoexigua pacifica* never swims or drifts at any stage of its life cycle (Komatsu *et al.*, 1990), its embryos and larvae should not be present in specimens collected from the sea with a plankton net. *A. pectinifera*, however, starts its planktonic life at the late blastula stage and then sinks to the bottom at the late brachiolaria stage. Therefore, we would expect that an ELISA system, with the K1 monoclonal antibody as the detecting antibody, could be used to quantify embryos and larvae of *A. pectinifera* in a complex planktonic sample.

We assessed the sensitivity of the assay system for embryos of *A. pectinifera* by testing graded quantities of embryos. Five thousand (5000) embryos, at each developmental stage, were boiled in 1 ml SDS sample buffer, the lysates were diluted 1:5000 to 1:40,000 in PBS, and a 100-μl aliquot was added to a well of a microtiter plate for ELISA. The absorbance decreased as development progressed, although on a per protein weight basis, the absorbance was nearly the same for all stages of the embryos examined. The protein content of a single oocyte, egg, or embryo at the mid-blastula stage (9 h after fertilization) was 0.30 μg; that of a single embryo at the mid-gastrula stage (24 h after fertilization) was 0.21 μg; and that of an early bipinnaria (72 h after fertilization) or mid-bipinnaria (96 h after fertilization) was 0.10 μg. The absorbance values changed linearly with concentration over the following

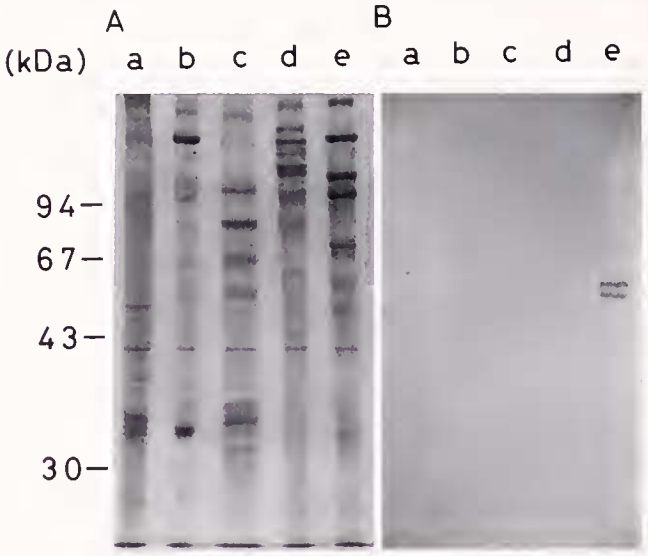

Figure 6. Immunoblots of eggs of *Scaphechinus mirabilis* (a), *Pseudocentrotus depressus* (b), *Hemicentrotus pulcherimus* (c), *Asterias amurensis* (d), and *Asterina pectinifera* (e). Eggs were solubilized in SDS sample buffer, and an aliquot of the sample solution equivalent to 10 μg protein was loaded onto each lane of SDS-10% polyacrylamide gel, electrophoresed, and then either stained with Coomassie brilliant blue (A) or blotted onto nitrocellulose and probed with the K1 antibody (B), as described in Materials and Methods.

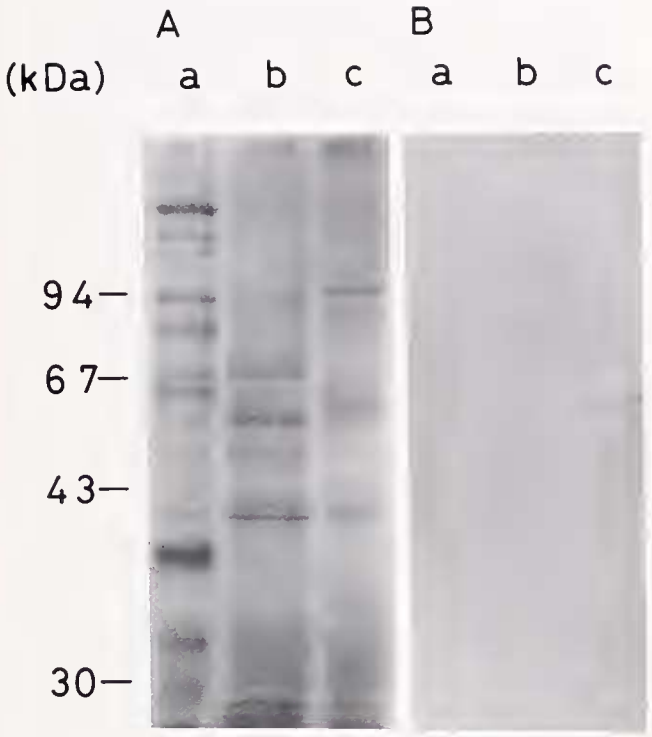

Figure 7. Immunoblots of *Asterina batheri* brachiolariae (a), *Asterina minor* brachiolariae (b), and *Asterina pectinifera* oocytes (c). Embryos and oocytes were solubilized in SDS sample buffer and an aliquot of the sample solution, equivalent to 5 μg protein, was loaded onto each lane of SDS-10% polyacrylamide gel, electrophoresed, and then stained with Coomassie brilliant blue (A) or blotted onto a nitrocellulose sheet (B). The sheet was probed with the K1 antibody, as described in Materials and Methods.

ranges: 0.0125 to 0.05 individuals per well for oocytes, eggs, and mid-gastrulae (24 h after fertilization); and 0.025 to 0.1 individuals per well for early bipinnariae (72 h after fertilization) and mid-bipinnariae (92 h after fertilization). It is not known at which stage of development the amount of the antigenic p58/56 in an embryo or larva becomes lower than the detection limit of the ELISA system with the K1 antibody. However, we succeeded in rearing five early brachiolaria of *A. pectinifera* (12 days after fertilization) by feeding bipinnariae in the laboratory. They were all loaded into a slot of an SDS-polyacrylamide gel, which was subjected, after electrophoresis, to immunoblot analysis. The sensitivity of the immunoblot analysis is approximately five times lower than that of the ELISA system used in this study. There were no immunoreactive bands on the slot (data not shown), suggesting that the amount of p58/56 present in a larva at the mid-bipinnaria stage decreased to less than 10% as development progressed to the early brachiolaria stage.

Although neither embryos nor larvae at various developmental stages could be quantified by the ELISA system, it could be used to estimate the minimum number of embryos or larvae present in a sample. We collected planktonic samples from 6 tons of water from the sublittoral zone of Hikonoura (1 or 3.5 m above the bottom of the sea, *ca.* 100 m off Sensui Island, *ca.* 34 23′N, 133 24′E) at high tide on November 22, 1987. The samples were spun down at 1000 × *g* for 10 min, and the precipitate was lysed in SDS sample buffer. Aliquots of the lysates were diluted 1:1000 in PBS, added to wells of a microtiter plate, and assayed by the ELISA system. The observed values were corrected with reference to a preparation containing a known number of *A. pectinifera* embryos at the mid-gastrula stage mixed with a standard amount of plankton lysate. The estimated numbers of *A. pectinifera* embryos or larvae in the three plankton samples collected separately from 6 tons each of water were 30, 130, and 130, assuming that the planktonic embryos were all mid-gastrulae. As described above, these figures are the minimum numbers of embryos and larvae present in the specimens, since the response of lysed larvae at later developmental stages was low compared with that of the mid-gastrulae, as shown in Figure 9.

Discussion

Mochizuki and Hori (1980) examined the phylogenetic relationship between five *Asterina* species by the enzyme

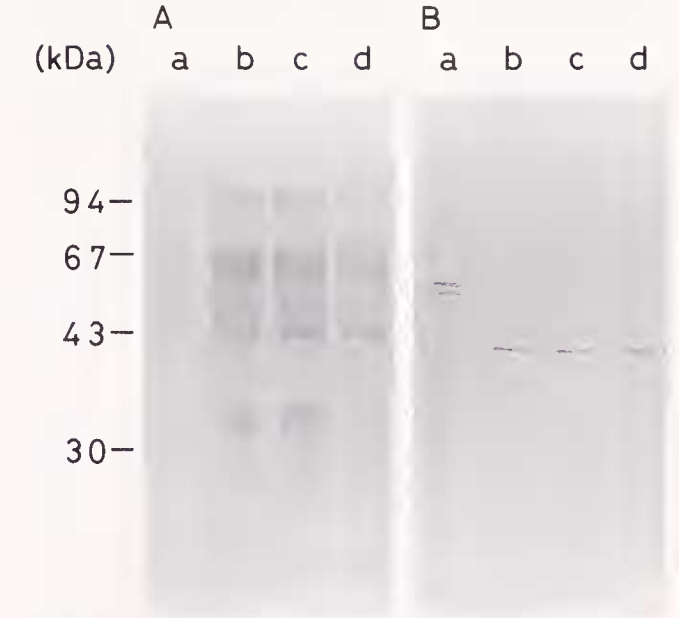

Figure 8. Immunoblots of *Asterina pectinifera* oocytes (a), and *Asterina pseudoexigua pacifica* early blastulae (b), wrinkled blastulae (c), and brachiolariae (d). Embryos and oocytes were solubilized in SDS sample buffer, and an aliquot of the sample solution, equivalent to five oocytes or embryos, was loaded onto each lane of SDS-10% polyacrylamide gel, electrophoresed, and then stained with Coomassie brilliant blue (A) or blotted onto a nitrocellulose sheet (B). The sheet was probed with the K1 antibody, as described in Materials and Methods.

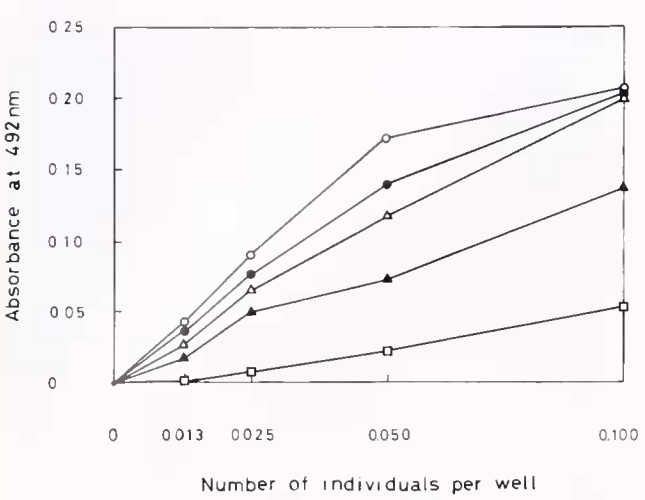

Figure 9. Measurement of antigen content in extracts of *Asterina pectinifera* oocytes (○), eggs (●), 1-day-old early gastrulae (△), 3-day-old late gastrulae (▲), and 4-day-old early bipinnariae (□) using an ELISA system with the K1 antibody. The method of measurement of antigen content is described in Materials and Methods.

inhibition test, using rabbit anti-*A. pectinifera* hexokinase antisera. The antisera inhibited the activity from hexokinase of *A. batheri, Asterina coronata japonica,* and *A. pseudoexigua pacifica* by 50–54% and that of *A. minor* by 30%. In light of these results, Mochizuki and Hori suggested that *A. pectinifera* and *A. minor,* but not the other *Astrina* species, belong to convergent lineages. Matsuoka (1981) arrived at a similar conclusion as a result of electrophoretic analysis of hexokinase and six other enzymes from five *Asterina* species. In our study, a peptide capable of recognizing the K1 monoclonal antibody, which is reactive to *A. pectinifera* p58/56, was found only in embryos and larvae of *A. pseudoexigua pacifica* among the *Asterina* species examined. Since little information is available regarding the function and structure of the peptides containing the K1 epitope, the reason for the phylogenetic variation of the peptides in these two species remains unclear.

So far, *A. pseudoexigua pacifica* has been found only in Sabiura, off Kushimoto in Wakayama Prefecture (Komatsu *et al.,* 1990). It is tiny (its body is only 5 mm long) and ovoviviparous. Development of this species, from fertilization to the completion of metamorphosis, occurs within the gonad. The juveniles, measuring approximately 0.5 mm in total length, crawl out of the gonad and pass through the gonoduct. According to the observation of Komatsu and Kano, made in July and August of 1972, the maximum number of juveniles released from a single adult was 1288 (Komatsu *et al.,* 1990). On the other hand, *A. pectinifera* is extremely abundant along the Japan Islands; an individual spawns more than 100,000 eggs, and it has embryonic and larval forms that live for a short time as members of the plankton community before moving into the benthic environment and undergoing metamorphosis. Therefore, the K1 antibody-reactive components detected by the ELISA assay in the plankton specimen were very unlikely to have been derived from embryos or larvae of *A. pseudoexigua pacifica.* Although we did not analyze the plankton specimens collected in the field by immunoblot assay, this assay can discriminate between embryos of *A. pseudoexigua pacifica* and those of *A. pectinifera,* according to the differences between molecular masses of the immunoreactive components. This enables *A. pectinifera* embryos and larvae in the mixed planktonic specimens to be identified unambiguously.

A. pectinifera differs from the other *Asterina* species in that the size and yolk content of its eggs are small, and that larval development, at both bipinnaria and brachiolaria stages, is indirect (Oguro and Komatsu, 1988). Therefore, this species can usually be identified accurately by microscopic examination of a larval specimen. However, there are huge numbers of diatoms in planktonic samples collected from water along the Japan Islands, and these make the detection of small numbers of starfish embryos and larvae almost impossible. Therefore, an immunological search for embryos and larvae in a complex plankton sample is particularly useful when their population is thin.

This study was aimed at determining the spawning season of *A. pectinifera* at specified regions along the coast of the Japan Islands. Ten percent of the total number of adult *A. pectinifera* caught on 29 October 1987, at Hikonoura off Sensui Island, contained ripe ovaries with full-grown oocytes. This fell to 3% on 15 November and to 1.7% on 19 December. These observations suggest that, at this sampling site, many adult *A. pectinifera* spawned at the beginning of November. For three years, spawning at this location has been observed to occur in November. Our results, obtained by immunological detection using an ELISA system, for *A. pectinifera* embryos or larvae in plankton samples collected at this location on 22 November 1987, agree well with these observations. However, inspection of the ovaries of adults collected from other areas along the coast of the Japan Islands revealed that the breeding season of this species is quite diverse, occurring in May in Tokyo Bay in Kanagawa Prefecture, June in Ago Bay in Mie Prefecture, July and August in the south-western part of the Inland Sea of Japan in Ehime Prefecture, September in Mutsu Bay in Aomori Prefecture, and November in the Sea of Yatsushiro in Kumamoto Prefecture. The spawning season in most other parts of Japan is, as yet, unknown. The spawning season in a specified region of the sea could be predicted by analyzing plankton specimens, sampled periodically, with the immunological assay method as described in this paper.

In summary, we have developed an assay system that detects embryos and larvae of *A. pectinifera* in complex biological specimens. The results of the this investigation suggest that immunological techniques are useful tools for taxonomic identification of eggs, embryos, or larvae in plankton populations.

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Literature Cited

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

Kanatani, H. 1973. Maturation-inducing substance in starfishes. *Int. Rev. Cytol.* **35:** 253–298.

Kano, Y. T., and M. Komatsu. 1978. Development of the sea-star, *Asterina batheri* Goto. *Dev. Growth Differ.* **20:** 107–114.

Komatsu, M. 1976. Wrinkled blastula of the sea-star, *Asterina minor* Hayashi. *Dev. Growth Differ.* **18:** 435–438.

Komatsu, M., Y. T. Kano, and C. Oguro. 1990. Development of a true ovoviviparous sea star, *Asterina pseudoexigua pacifica* Hayashi. *Biol. Bull.* **179:** 254–263.

Komatsu, M., Y. T. Kano, H. Yoshizawa, S. Akabane, and C. Oguro. 1979. Reproduction and development of the hermaphroditic sea-star, *Asterina minor* Hayashi. *Biol. Bull.* **157:** 258–274.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Matsuoka, N. 1981. Phylogenetic relationships among five species of starfish of the genus, *Asterina:* an electrophoretic study. *Comp. Biochem. Physiol.* **70B:** 739–743.

Mochizuki, Y., and S. Hori. 1980. Immunological relationships of starfish hexokinases: phylogenetic implication. *Comp. Biochem. Physiol.* **65B:** 119–125.

Oguro, C., and M. Komatsu. 1988. Asteroidea. Pp. 339–356 in *Development of Invertebrates,* K. Dan, K. Sekiguchi, H. Ando and H. Watanabe, eds. Baifukan, Tokyo. (in Japanese)